CIRCADIAN REGULATION OF TRANSLATION IN NEUROSPORA CRASSA

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Circadian regulation of translation in *Neurospora crassa*

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The circadian clock controls daily rhythms in messenger RNA (mRNA) accumulation of up to 40% of the eukaryotic genome. In addition, in the mouse liver, about 50% of proteins that cycle in abundance are produced from mRNAs that are constitutively expressed, indicating a role for the clock in the regulation of translation. While the basic mechanisms of circadian transcriptional control are known, little is understood about how the clock controls mRNA translation. We discovered that the clock in *Neurospora crassa* regulates the activity of eukaryotic elongation factor 2 (eEF2), a necessary component of translation elongation. These data suggested that clock regulation of protein abundance is dependent on rhythms in the activity of eEF2. To begin to test this idea, we used RNA-sequencing and ribosome profiling to determine which mRNAs and proteins cycle in abundance in *N. crassa* under control of the clock. For most genes, the peak ribosome occupancy of the mRNA coincided with the peak in mRNA levels. However consistent with clock regulation of translation, our preliminary data suggests that for some genes, rhythms in ribosome occupancy occurred on mRNAs that were constitutively expressed. My goal is to first validate the preliminary results for candidate genes that cycle only at the ribosome occupancy level, and then determine if the protein rhythms are dependent of clock control of eEF2 activity.

DEDICATION

I would like to dedicate this project to Toni Szutkowski. Your support has been a huge help.

ACKNOWLEDGMENTS

I would like to give a special thanks to Stephen Caster and Dr. Bell-Pedersen. Their guidance and patience has been instrumental in both the lab and in my life. I couldn't be part of a better lab, and I appreciate it every day. I would also like to thank all the members of the Dr. Bell-Pedersen Lab for being patient with my questions and always having time to teach me new things.

CHAPTER I

INTRODUCTION

The circadian clock is an evolutionarily conserved time-keeping mechanism that, through the regulation of rhythmic gene expression, coordinates the physiology of an organism with the daily environmental cycle (1). External cues, particularly light signals, entrain clocks to 24-h environmental cycles, but in constant environmental conditions, the clock will free-run with its endogenous period close to 24 -h. Because virtually all aspects of human physiology and behavior are linked to the clock, abnormalities in the circadian system are associated with a wide range of diseases such as sleep disorders, cardiovascular disease, metabolic syndrome, and cancer (2). In addition, the clock controls temporal aspects of drug metabolism and vulnerability to cytotoxic agents (3). Thus, knowing what genes and proteins are regulated by the clock, and the mechanisms of this regulation, are necessary to understand clock-associated diseases and rhythmic drug metabolism.

Because clock mechanisms are conserved, we can use genetically tractable organisms to do experiments to understand how the clock works at a molecular level. To date, one of best-studied organisms for circadian rhythms is the filamentous fungus *Neurospora crassa*. In *N. crassa,* the circadian clock consists of a molecular oscillator that involves two positive elements called WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), which form the WC complex (WCC) and the negative component FREQUENCY (FRQ) (4). The WCC functions as both a blue light photoreceptor and transcription factor. WCC is activated in the morning, and binds to the *frq* promoter to turn on *frq* transcription. FRQ protein accumulates and

heterodimerizes with FRQ interacting RNA helicase (FRH). The FRQ/FRH complex binds to the WCC, and recruits several different kinases. These kinases phosphorylate WC-1, which stabilize and inactivate it. Over time FRQ protein is degraded and WC-1 dephosphorylates and becomes active again to restart the molecular cycle the next day. In addition to binding of the *frq* promoter, the WCC binds to the promoters of up to 200 genes leading to their rhythmic expression, and that results in overt rhythmicity (5). The clock mechanism is conserved throughout eukaryotes (6). Thus, what we learn about the clock in the simple model organism *N. crassa* can help us to better understand how the clock works in humans, and what goes wrong when we live against our clock, such as during shift work.

Most research on clock control of rhythmic gene expression has been at the transcriptional level (7), but there is increasing evidence that the clock regulates gene expression at the translational level (6, 8). In *N. crassa* cells, evidence for circadian control of translation came from studies demonstrating clock control of the osmo-sensing (OS) mitogen-activated protein kinase (MAPK) pathway. In constant environmental conditions, the WCC binds rhythmically to the promoter of *os-4*, the mitogen-activated protein kinase kinase kinase (MAPKKK), which leads to rhythmic OS-4 protein accumulation. This in turn leads to OS-4 rhythmically signaling through the MAPK pathway to the terminal MAPK OS-2, resulting in rhythmic phosphorylation of OS-2 (9). Phosphorylation activates OS-2, which allows it to interact with transcription factors, chromatin modifying proteins, and other kinases. One of these kinases, RCK-2, regulates the phosphorylation of translation elongation factor eEF-2. When eEF-2 is phosphorylated, translation elongation stops. These data led us to hypothesize that rhythmic control of eEF-2 phosphorylation by the clock leads to rhythmic translation of at least some *N. crassa* mRNAs.

Using RNA-seq and ribosome profiling, we identified several candidate gene mRNAs that are differentially regulated at the translational level. Two of these candidate genes, oxalate decarboxylase (*oxdC*) and glutathione S-transferase-3 (*gst-3*), were chosen for validation and further study. Based on our genome-wide studies, in wild type (WT) cells grown in constant environmental conditions, *oxdC* mRNA levels and ribosomal occupancy are rhythmic, but their cycling is anti-phase. The other candidate gene, *gst-3*, has constitutive levels of mRNA, but rhythmic ribosomal occupancy. The goal of this study is to use different methods to verify that *oxdC* mRNA levels peak anti-phase to its ribosomal occupancy, and that *gst-3* mRNA is constitutive with rhythmic ribosomal occupancy. These data will allow us to determine if the clock is regulating translation through rhythmic control of eEF-2 activity, and if we are able to validate the genome-wide data, the extent of, and biological pathways under, this control.

CHAPTER II

MATERIALS AND METHODS

The major goal of this project is to determine if the clock in *N. crassa* controls rhythmic translation, specifically through RCK-2 and its subsequent rhythmic phosphorylation of eEF-2. Using ribosome profiling and RNA-seq (10, 11) from wild-type (WT) we found that about 10% of expressed genes have mRNAs that accumulate constitutively, but that have rhythms in ribosome occupancy. In addition, we identified some genes with rhythmic mRNA levels and rhythmic ribosome occupancy, but that peak out of phase. These data suggested that translational control of gene expression by the clock might be important in phase determination of protein levels for some genes. My goal is to select one gene in each category to help validate the genome-wide data. One candidate gene is *gst-3*, which appears to have constitutively expressed mRNA, but rhythmic mRNA ribosomal occupancy. The other candidate gene, *oxdC*, appears to have rhythmic mRNA accumulation that peaks during the subjective daytime in constant conditions, but mRNA ribosomal occupancy that peaks during the subjective night.

To assay protein levels of GST-3 and OXDC *in vivo*, 3-way PCR was used as previously described (3) to generate a translational fusion of the candidate genes to luciferase. Briefly, the reporter firefly luciferase (luc) gene, coupled with *hph* (a gene that encodes for hygromycin resistance) was fused to the 3' end of either *gst-3* or *oxdC* coding region, deleting the stop codon. The resulting construct was then coupled with the 3' un-translated region (UTR) of *gst-3* or *oxdC,* to allow for targeting the construct to the native locus and generating an in-frame fusion between the coding regions of gst-3/oxdC to the coding region of luc. The constructs were

validated by sequencing, and transformed into WT, Δ*frq*, and Δ*rck-2* cells, as previously described (12). To examine mRNA levels of *oxdC* and *gst-3*, the promoters of the genes for fused to luc by PCR and flanked by the 5' and 3' ends of the *his-3* gene for targeting the fusion constructs to the *his-3* locus. For the constructs, I amplified 1) a portion of the *his-3* gene; 2) the promoter region of *gst-3* or *oxdC*; 3) the luc gene; and 4) the 3' end of *his-3*. The primers were designed to have ends that are homologous to the neighboring region as previously described (12). The fragments were cloned into pCRTM-Blunt vector (#K2700-20, Thermo Fisher Scientific Inc., Waltham, MA, USA) and validated by sequencing. Once validated, the plasmid was linearized using a restriction digest, and transformed into WT, Δ*frq*, and Δ*rck-2* cells by electroporation (13). The translational fusion transformants were screened for their ability to grow on media with hygromycin, and the transcriptional fusion transformants on media lacking histidine, followed by assaying the cells for luciferase activity. PCR was used to validate that the constructs were targeted to the correct locus. Subsequently, luciferase fusion contrstucts were analyzed with a top count that measured luciferase luminosity. The WT fusion strain (#937) were crossed with Δ*frq* or Δ*rck-2* cells using standard techniques.

CHAPTER III

RESULTS

In *N. crassa* cells, about half of the genome is controlled by the clock at the level of transcript abundance (11). However, little is known about circadian clock regulation of translation. To determine the extent of clock control of translation we used ribosome profiling, which maps ribosomes to mRNAs and serves as an indicator of translation rates (14), coupled

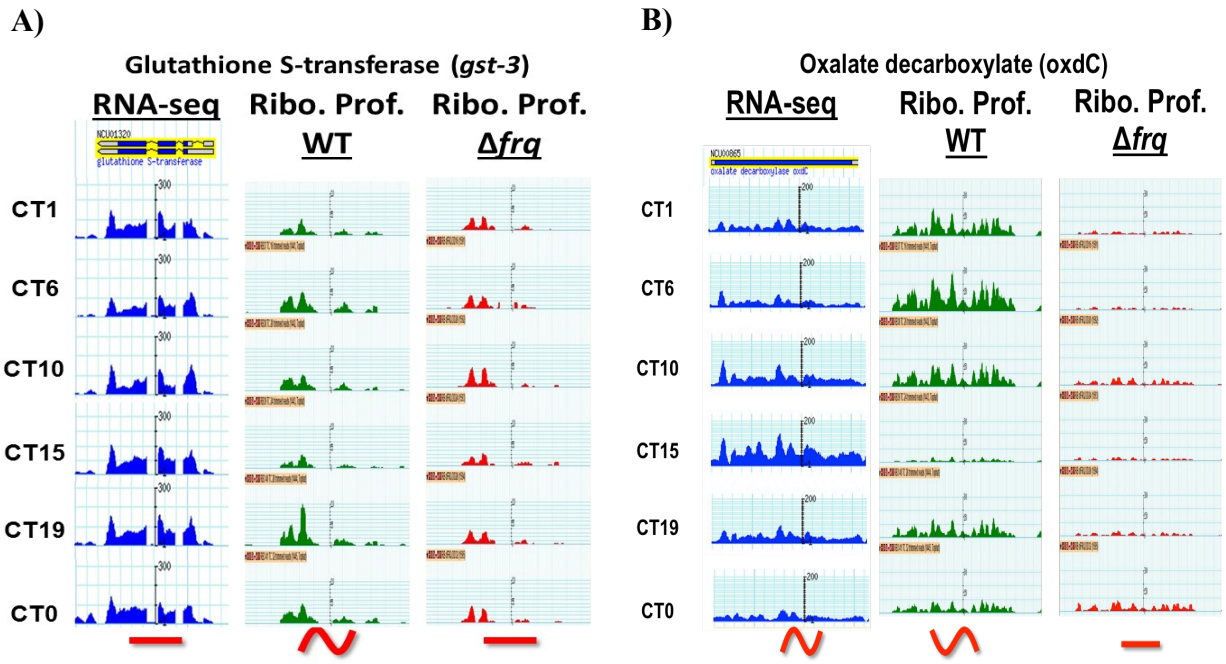

Figure 1. The blue column depicts levels of mRNA. A) mRNA levels of *gst-3* remain constant but the ribosomal occupancy is rhythmic (green column). However in a clock mutant strain (red column), the ribosomal occupancy is arrhythmic. B) The mRNA for *oxdC* is cycling and the ribosomal occupancy is also rhythmic. However the rhythms in ribosomal occupancy are anti-phase to the mRNA levels. In the clock mutant strain the rhythms are abolished.

with RNA-seq to measure the levels of individual mRNAs. Using ribosome profiling and transcriptome analysis we have compared mRNA levels to ribosomal occupancy in WT and Δ*frq* (a clock mutant strain) cells isolated at different times of the day.

Our preliminary data show that about 10% of mRNAs have rhythmic ribosomal occupancy and constitutive mRNA levels. For example, *gst-3* mRNA accumulates constitutively during the day; however, ribosome binding to *gst-3* mRNA is rhythmic in WT cells peaking at circadian time (CT) 19 (Figure 1A). Circadian time is used to normalize biological time in strains with different endogenous period lengths to 24 circadian hours per cycle, where CT0 represents dawn and CT12 represents dusk. The rhythm in ribosome occupancy was abolished in the clock mutant Δ*frq*, confirming regulation by the clock. Interestingly, in addition to rhythms in ribosome occupancy from constitutive mRNAs, we also identified genes where the peak phase of the rhythm between mRNA levels and ribosome occupancy is anti-phase. One example is *oxdC* (Figure 1B). The levels of *oxdC* mRNA peaks in the night at CT15, whereas *oxdC* mRNA ribosome occupancy peaks at noon, CT6. These data support the idea that the clock regulates rhythmic translation of mRNAs that accumulate constitutively, and that clock regulation of translation can have a major effect on the time of peak protein levels.

To first validate the ribosome profiling data, a translational fusion of GST-3 and OXDC to a luciferase reporter was generated and was used to replace the WT endogenous gene. We found that both GST-3::LUC and OXDC::LUC cycle with a peak that is near the time of the peak in the ribosome profiling results (Figure 2). For GST-3, ribosome occupancy peaks at CT19 (Figure 1) and luciferase activity peaks at DD24 (Figure 2), which represents CT 15. For OXDC, ribosome occupancy peaks at CT6 (Figure 1), and luciferase activity peaks at DD 22, which represents CT 12. Experiments are in progress to validate the RNA-seq data for these genes.

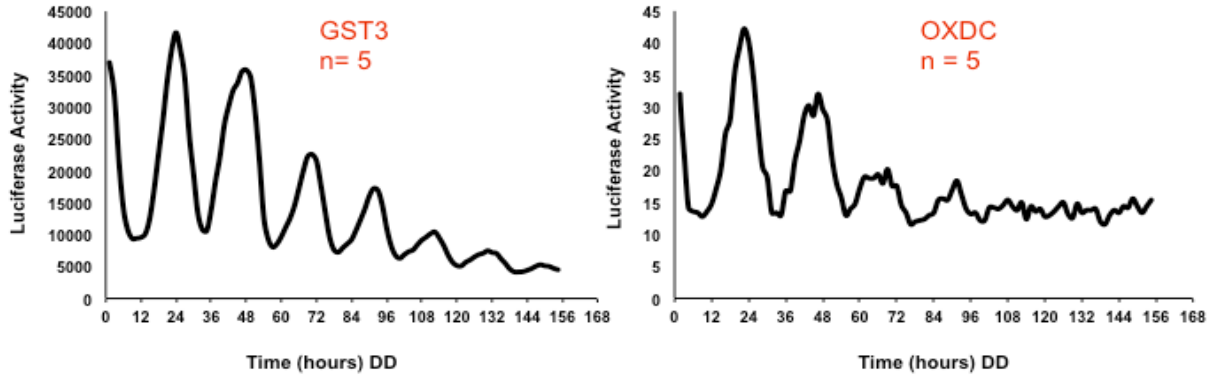

Figure 2. Luciferase activity cycles from cells containing GST-3 and OXDC Luciferase fusions, with a peak that is consistent with ribosomal profiling data. The average luciferase activity levels from 5 cultures grown in the dark (DD) sampled every 90 min over 6.5 days is plotted.

We recently discovered that the clock controls the activity of eEF-2 through the eEF-2 kinase RCK-2. If clock regulation of eEF-2 plays a role in circadian translational control, we predict that the rhythms in GST-3::LUC and OXDC::LUC will be abolished in RCK-2 deletion cells. To begin to test this idea, I crossed the GST-3::LUC strain (#937) to a Δ*rck-2*::hygR strain (#1116). From this cross, the possible progeny I could obtain are the two parental strains, the desired GST-3::LUC in the Δ*rck-2*;hyg background, and its WT sibling (*gst-3*$^{+}$, *rck-2*$^{+}$). From 48 spores picked from the cross, 30 germinated (63%). To determine if the progeny contained the *rck-2* deletion, I examined if they were resistant to hygromycin. As expected about half of the 30 progeny grew on slants containing 200 μg of hygromycin. To then determine if any of the 13 hygR stains contained GST-3::LUC, diagnostic PCR was used (Figure 3). Forward and reverse primers 1 and 2 would amplify a 5.5 kb fragment from the intact *rck-2* locus, and a 4.5 kb

fragment from Δ*rck-2*, and primers 1 and 3 would only amplify a product (2.1 kb) for Δ*rck-2* (Figure 3 A&B). Out of the 13 progeny that were hygromycin resistant, 1 progeny (#7) was not validated by PCR (Figure 4A&B).

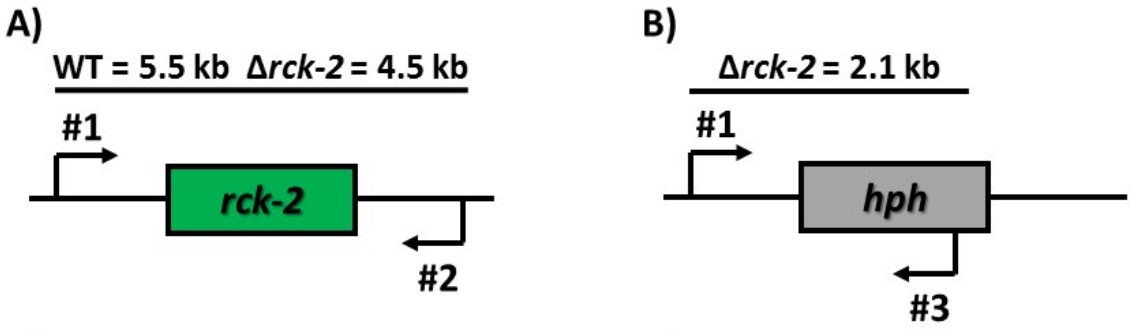

Figure 3. In a WT strain using primer set described in A, we would expect amplification fragments 5.5 kb in size for only WT background strains. In the second primer set (B), we would get a 2.1 kb fragment amplification of our knockout but not a WT strain.

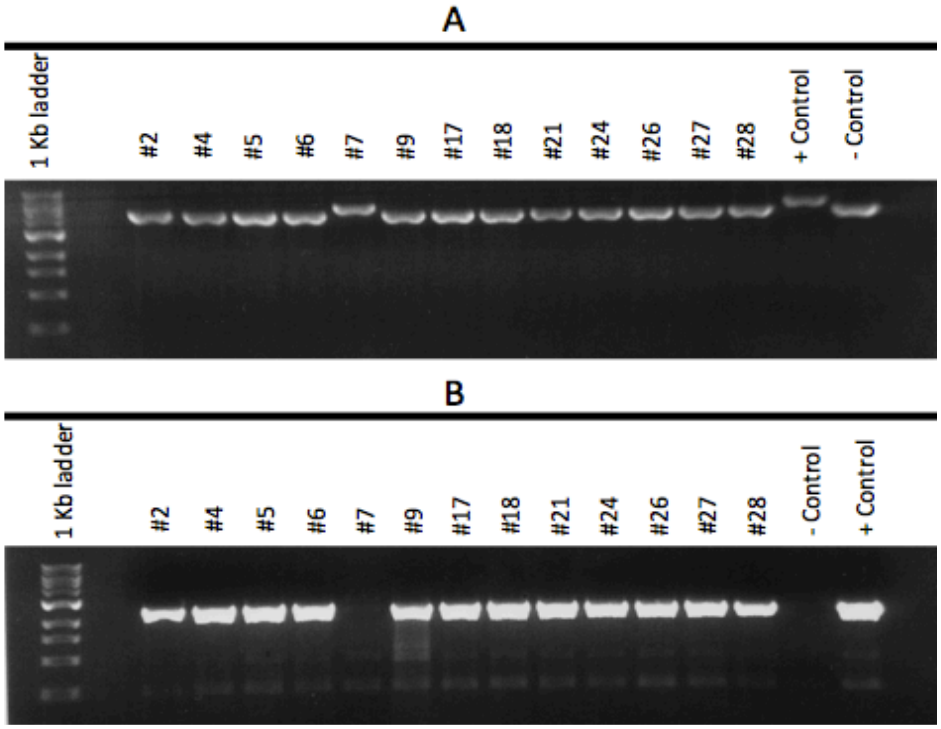

Figure 4. A) We expect to see a 5.5 kb fragment for cells containing the *rck-2* gene and a 4.5 kb fragment for cells containing the knockout gene. All samples except for #7 had a 4.5 kb fragment, indicating the presence of the Δ*rck-2* deletion. B) We observed amplification of the expected size fragment in all but sample #7, indicating all other samples have the *hph* gene conferring hygromycin resistance. 1Kb ladder sizes in descending order are 10, 8, 6, 5, 4, and 3Kb.

The 12 progeny strains were then examined for luciferase activity; however, none of them had any luciferase activity, whereas the control parental strain #937 had luciferase activity. In

conclusion the 12 progeny were not of the desired *gst-3::luc*; Δ*rck-2::hygR* genotype. The most likely explanation for the inability to recover the correct strain is that the *gst-3::luc* strain (#937) is a heterokaryon which would reduce the likelihood of obtaining the desired progeny. The cross was therefore repeated using a homokaryon of *gst-3::luc*, yielding the desired strain.

Comparison of luciferase levels from the *gst-3::luc* fusion in WT and Δ*rck-2* cells revealed that RCK-2 is necessary for normal rhythms of GST-3. These data are consistent with a role for clock control of the activity of eEF-2, via phosphorylation by RCK-2, in controlling translation.

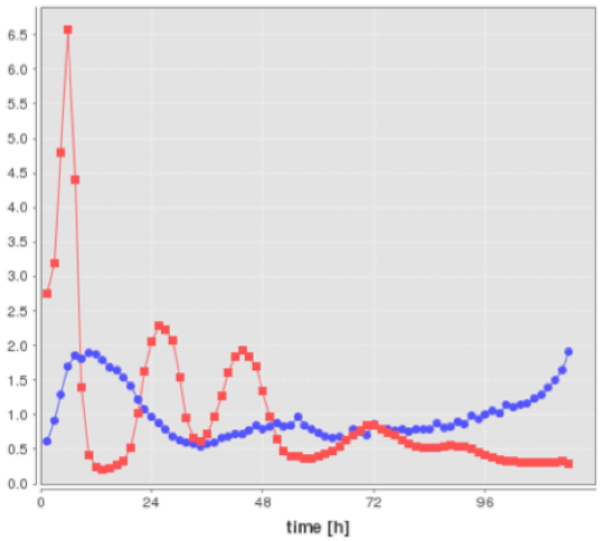

Figure 5. The red line represents GST-3::LUC protein levels in a WT background and the blue line represents GST-3::LUC protein levels in Δ*rck-2* background. The levels of GST-3::LUC are rhythmic in WT cells, but arrhythmic in Δ*rck-2* cells**.**

CHAPTER IV

CONCLUSIONS

While evidence in several model systems indicates circadian clock control of translation exists, the mechanisms for how this regulation occurs was not known in any organism. We found that the circadian clock controls protein levels through rhythmic phosphorylation and inactivation of eEF-2. Because translation is the most energetically demanding process in the cell, linking the timing of translation to the time of peak energy production would be expected to provide an advantage to the organism. Furthermore, understanding how protein levels are regulated can provide new insights into medicine, including the timing of chemotherapy. Chronochemotherapy is the use of chemotherapy at specific times of the circadian cycle when normal cells are not dividing, which increases the efficacy of the chemotherapy and decreases the adverse side-affects (15). If we can identify the mechanism for clock control of translation, and identify which proteins are under this control, then we can effectively identify targets for potential drugs and treatment for the betterment of people with cancer.

In an attempt to demonstrate clock regulation of mRNA at the translational level, I focused first on validating initial RNA sequencing and ribosomal profiling data. To do so, we identified two potential candidates with differential regulation when comparing mRNA levels to mRNAs being actively translated. The two candidate genes studied were *gst-3* and *oxdC*. We generated translational fusions of the two genes with luciferase using 3-way PCR (12). These fusions were assayed for luciferase rhythms (Figure 2). Both translational fusions of GST3 and OXDC showed rhythms consistent with the initial ribosomal profiling data. Subsequently the fusions

were then crossed into Δ*rck-2* cells to determine if clock control of translation required RCK-2 and eEF-2. However, only the GST-3::LUC fusion was successfully crossed to Δ*rck-2* cells. Initially, this cross failed because one of the parent strains was a heterokaryon, effectively reducing the probability of obtaining the desired genotype from 50% to 25%. Subsequent crosses using a homokaryotic strain of GST-3::LUC was successful, and allowed us to test if RCK-2 is necessary for the GST-3::LUC rhythms. We found that in the absence of RCK-2, GST3::LUC accumulated arrhythmically. RCK-2 is a kinase that phosphorylates and inactivates eEF-2. Therefore, these data suggest that the rhythms in accumulation of GST-3::LUC are due to rhythmic phosphorylation of eEF-2, providing new insights into the mechanism of circadian control of translation. Future experiments would be to obtain the OXD-C:LUC fusion in Δ*rck* cells to determine if rhythms in eEF-2 activity impact the phase of rhythmic protein levels. Finally, positive validation of certain candidates from the ribosome profiling and transcriptome data will provide a level of confidence that our genome-wide data accurately reflects what is happening in the cell, and will provide the opportunity to discover how many genes and what biological pathways are regulated at the level of translation through eEF-2 activity.

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